Molecular Predictors of Sensitivity to the MET Inhibitor PHA665752 in Lung Carcinoma Cells

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Background: No comprehensive data are available on the molecular predictors of sensitivity to MET inhibitor in lung carcinomas.

Methods: We examined the efficacy of the MET inhibitor PHA665752 in 41 cell lines of non-small lung carcinoma to determine whether sensitivity to the MET inhibitor is correlated with the (1) genetic statuses of MET, epidermal growth factor receptor (EGFR), human epidermal growth factor receptor 2, and KRAS, (2) MET phosphorylation and its downstream signaling pathways, or (3) epithelial-mesenchymal transition.

Results: Of the 41 cells, 8 were highly or intermediately sensitive to PHA665752, and the remainder were PHA665752 resistant. The sensitive cells ($n = 8$) included not only 4 of 4 MET-amplified cell lines but also 2 of 11 KRAS-mutated cell lines and 1 of 6 EGFR-mutated cell lines. Unlike the MET-amplified cell lines, both the MET-mutated cell lines were PHA665752 resistant. High phospho-MET was not restricted to the four MET-amplified cell lines. To the contrary, it was also found in 9 of 37 MET-nonamplified cell lines, including 3 of 6 EGFR-mutated cell lines and 4 of 11 KRAS-mutated cell lines. High phospho-MET was correlated with PHA665752 sensitivity in the entire panel of cell lines, especially in the KRAS-mutated cells. The AKT and ERK pathways in the high phospho-MET cell lines were dependent on MET activation in MET-amplified and KRAS-mutated cells but not in EGFR-mutated and human epidermal growth factor receptor 2-amplified cells.

Conclusions: MET amplification is an excellent predictor of PHA665752 sensitivity but not the sole predictor. High phospho-MET and dependence of the AKT and ERK pathways on MET activation may predict sensitivity to PHA665752, especially in KRAS-mutated cell lines.

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Lung cancer is the leading cause of cancer mortality in developed countries. Molecularly targeted therapy is a new therapeutic modality now under intense investigation.[1] The epidermal growth factor receptor (EGFR) inhibitors gefitinib and erlotinib have already shown promising results for patients with lung adenocarcinomas.[2] Molecular analyses have demonstrated that EGFR-mutated tumors are commonly sensitive to these EGFR inhibitors, whereas KRAS-mutated tumors are most often resistant.[3]

MET is another receptor-type tyrosine kinase overexpressed and activated in a subset of lung adenocarcinoma tissues and cell lines.[4–6] Earlier studies have reported that a small molecule MET inhibitor, PHA665752, inhibits the growth of various cancer cell lines,[7–10] and that MET amplification may be useful for identifying cell lines sensitive to the MET inhibitor.[8,10] Yet, the number of lung carcinoma cell lines tested in these previous studies has been rather limited. In an examination of 40 human cancer cell lines by Smolen et al.,[8] MET amplification was harbored in 1 of 4 gastric cancer cell lines versus 0 of 12 lung cancer cell lines. In the study by Lutterbach et al.,[10] two MET-amplified lung cancer cell lines with high MET and high phospho-MET expressions were compared with seven non-MET–amplified lung cancer cell lines with low MET and low phospho-MET expressions. According to their results, the former were sensitive to MET inhibitor whereas the latter were resistant to it. Although these results are clear, the nine cell lines analyzed may not fully represent the diversity of human lung cancer.

In this study, we performed a comprehensive analysis of a large panel of lung carcinoma cell lines and sought to identify molecular determinants of sensitivities to the MET inhibitor. Specifically, we asked whether sensitivities to the MET inhibitor are correlated with (1) the genetic statuses of MET, EGFR, human epidermal growth factor receptor 2 (HER2), and KRAS; (2) the phosphorylation of MET and its downstream signaling pathways; or (3) epithelial-mesenchymal transition (EMT).

MATERIALS AND METHODS

Cell Lines and Medium

We used 41 non-small lung cancer cell lines. The sources and histologic types of these cell lines are detailed in Figure 1.

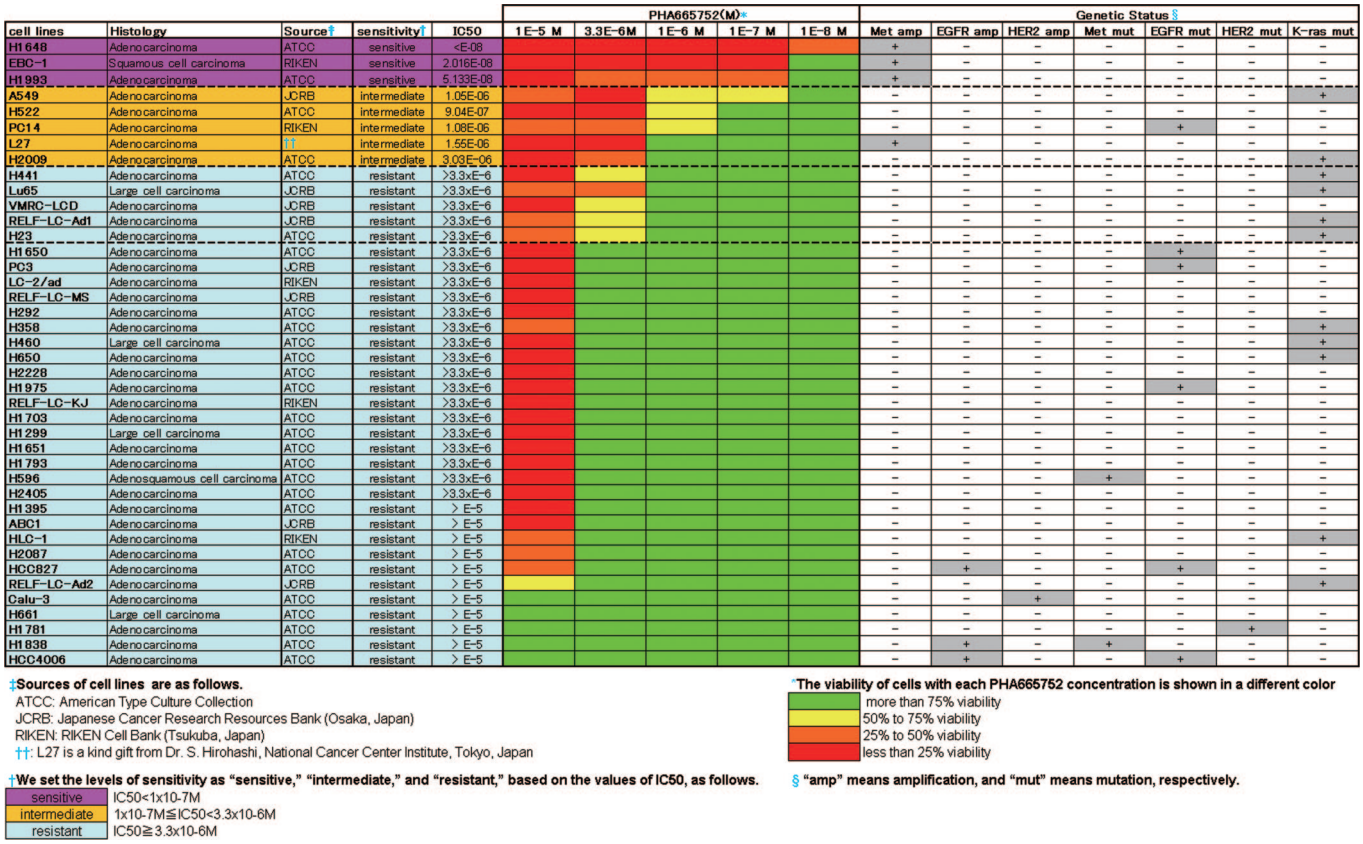

FIGURE 1. Sensitivity of 41 non-small cell lung cancer cell lines to PHA665752 and their genetic statuses. Left panel shows cell line names, histologies, sources, sensitivities, and the values of IC_{50}. Middle panel shows the viability of cells with each PHA665752 concentration in a different color. Right panel shows the genetic statuses of 41 cell lines. Details are shown at the bottom of the figure. L27 was described in a previous study.[11]

TABLE 1. Antibodies Used in Western Blot Analysis

Antibodies	Clone	Sources
c-MET	Rabbit polyclonal	IBL (Gunma, Japan)
phospho-c-MET (Y1234/Y1235) (#3126)	Rabbit polyclonal	Cell Signaling Technology (Danvers, MA)
phospho-ERK (Tyr202/Tyr204) (#9101)	Rabbit polyclonal	Cell Signaling Technology (Danvers, MA)
phospho-AKT (Ser473) (#9271)	Rabbit polyclonal	Cell Signaling Technology (Danvers, MA)
Anti-rabbit IgG peroxidase conjugate		Amersham (Arlington Heights, IL)

All cell lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum, glutamine, and antibiotics in a humidified atmosphere with 5% CO_2 and 95% air.

Antibodies

The sources of the antibodies used in this study are provided in Table 1.

DNA Sequencing

The DNA was extracted from cell lines by standard procedures. The polymerase chain reaction (PCR) primers and conditions for amplifying and sequencing exon 18 through exon 21 of the EGFR gene are described in the previous literature.[12] The mutation resulting in deletion of exon 14 of MET was screened by amplifying cDNA encompassing exon 14 of MET and examining the PCR product size on gel electrophoresis. The PCR primers and the conditions for mutational analysis of KRAS (codons 12, 13, and 61), MET (exons 2 and 3 encoding Sema domain), and HER2 (exons 19 and 20) are available upon request. The PCR products were sent to Macrogen Inc. (http://www.macrogen.com/eng/macrogen/macrogen_main.jsp) for sequencing.

Gene Expression Profile and Single Nucleotide Polymorphism Array Analysis

A comprehensive gene expression analysis was performed using an oligonucleotide microarray (GeneChip Human Genome U133A, Affymetrix, Santa Clara, CA) as described previously.[13] Single nucleotide polymorphism array (Affymetrix human mapping 50K XbaI array) analysis was

performed using GIM (Genome Imbalance Map) algorithm as described previously.[14]

Western Blot Analysis

Western blot analysis was performed as described previously.[6] The luminescence signal was detected and analyzed with the ChemiDoc XRS image analysis system (Bio-Rad, Tokyo, Japan).

Cell Proliferation Assay

Cell viability was measured by the Cell Counting Kit-8 assay (Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Cells ($4-8 \times 10^3$ cells) were plated in each well of 96-well microtiter plates. After 24 hours, PHA-665752 (kindly provided by Dr. J. Christensen, Pfizer) was added to each well to a final concentration of 10, 3.3, 1, 0.1, 0.01, and 0.001 μM. Cells were incubated for additional 4 days at 37°C. The absorbance of each well at 450 nm was measured with a reference at 630 nm using a Bio-Rad model 680XR microplate reader (Bio-Rad, Hercules, CA). IC_{50} value was calculated using a software GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA).

Statistical Analysis

The Mann-Whitney *U* test was used to evaluate the correlation between drug sensitivity and the expressions of MET, phospho-MET, and EMT markers and the correlation between genetic status and the expressions of EMT markers. The results were considered significant if the *p* value was less than 0.05. All statistical calculations were performed using the StatView computer program (Abacus Concepts, Berkeley, CA).

RESULTS

Sensitivity of Non-small Cell Lung Cancer Cell Lines to PHA665752, a MET Tyrosine Kinase Inhibitor

We examined a panel of 41 non-small cell lung cancer cell lines to assess their sensitivity to PHA665752 (Figure 1). We set the levels of sensitivity as "sensitive," "intermediate," and "resistant," defining them as $IC_{50} < 1 \times 10^{-7}$ M, 1×10^{-7} M $\leqq IC_{50} < 3.3 \times 10^{-6}$ M, and $IC_{50} \geqq 3.3 \times 10^{-6}$ M, respectively. As a result, 3 cell lines (H1648, EBC1, and H1993) were deemed to be sensitive, 5 cell lines (A549, H522, PC14, L27, and H2009) intermediate, and the remaining 33 cell lines resistant. Figure 2 shows a representative dose-response curve for each group of cell lines.

Figure 1 also shows the genetic statuses of the 41 cell lines. MET amplification was found in 4 cell lines (H1648, EBC1, H1993, and L27); MET mutation in 2 cell lines (H596 and H1838); EGFR mutation in 6 cell lines (HCC827, HCC4006, PC3, PC14, H1650, and H1975); EGFR amplification in 3 cell lines (HCC827, H1838, and HCC4006); HER2 mutation in 1 cell line (H1781); HER2 amplification in 1 cell line (Calu3); and KRAS mutation in 11 cell lines (A549, H23, H358, H441, H460, H650, H2009, HLC-1, Lu65, RELF-LC-Ad1, and RELF-LC-Ad2). The MET amplification, MET mutation, EGFR mutation, HER2 mutation, HER2 amplification, and KRAS mutation were all mutually exclusive, whereas EGFR amplification coexisted with either EGFR mutation or MET mutation. All the sensitive cell lines harbored MET amplification, and all four cell lines with MET amplification showed some sensitivity to PHA665752. This suggests that MET amplification is an excellent molecular predictor of susceptibility to the MET inhibitor. However, Figure 1 also shows that MET amplification was not the sole determinant of sensitivity to PHA665752, because four of the five cell lines showing intermediate sensitivity harbored no MET amplification. It was also of note that H596 and H1838, the two cell lines harboring MET mutation, were resistant to PHA665752.

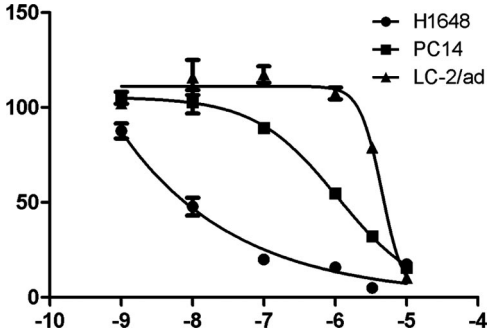

FIGURE 2. Dose-response curves of representative cell lines of each group: H1648 (sensitive), PC14 (intermediate), and LC-2/ad (resistant). The *x* axis indicates the $\log_{10}$(concentration of PHA665752) and the *y* axis indicates the %cell viability = (mean absorbance in test wells)/(mean absorbance in control well) $\times$ 100.

The distribution of PHA665752 sensitivity differed among KRAS-mutated cell lines. Two of the KRAS-mutated cell lines (A549 and H2009) showed intermediate sensitivity, four others (H441, Lu65, RELF-LC-Ad1, and H23) were resistant but showed some response at 3.3×10^{-6} M, and the remaining five were highly resistant with minimal inhibition at 3.3×10^{-6} M.

We were interested to note that all but one cell lines harboring genetic abnormalities of the ErbB family showed some level of resistance to PHA665752. This resistance was especially strong ($IC_{50} > 10^{-5}$ M) in the cell lines harboring EGFR amplification, HER2 mutation, and HER2 amplification. The one exception, PC14 harbored EGFR mutation, yet showed an intermediate sensitivity.

Correlation Between Constitutive Activation of MET and Sensitivity to PHA665752

Next, we examined the expressions of MET and phospho-MET in 41 cell lines by Western blot analysis. MET was strongly expressed in 16 cell lines (MET/beta-actin >2.0 arbitrary units, red bar in Figure 3*A*) and activated in 13 cell lines (p-MET/beta-actin >2.0 arbitrary units, red bar in Figure 3*B*). All four cell lines harboring MET amplification expressed MET and phospho-MET strongly. We confirmed a good correlation between MET expression and phospho-MET expression in 41 cell lines by correlation analysis (Figure 3*E*, correlation coefficient = 0.845). The levels of MET and phospho-MET expression were significantly higher

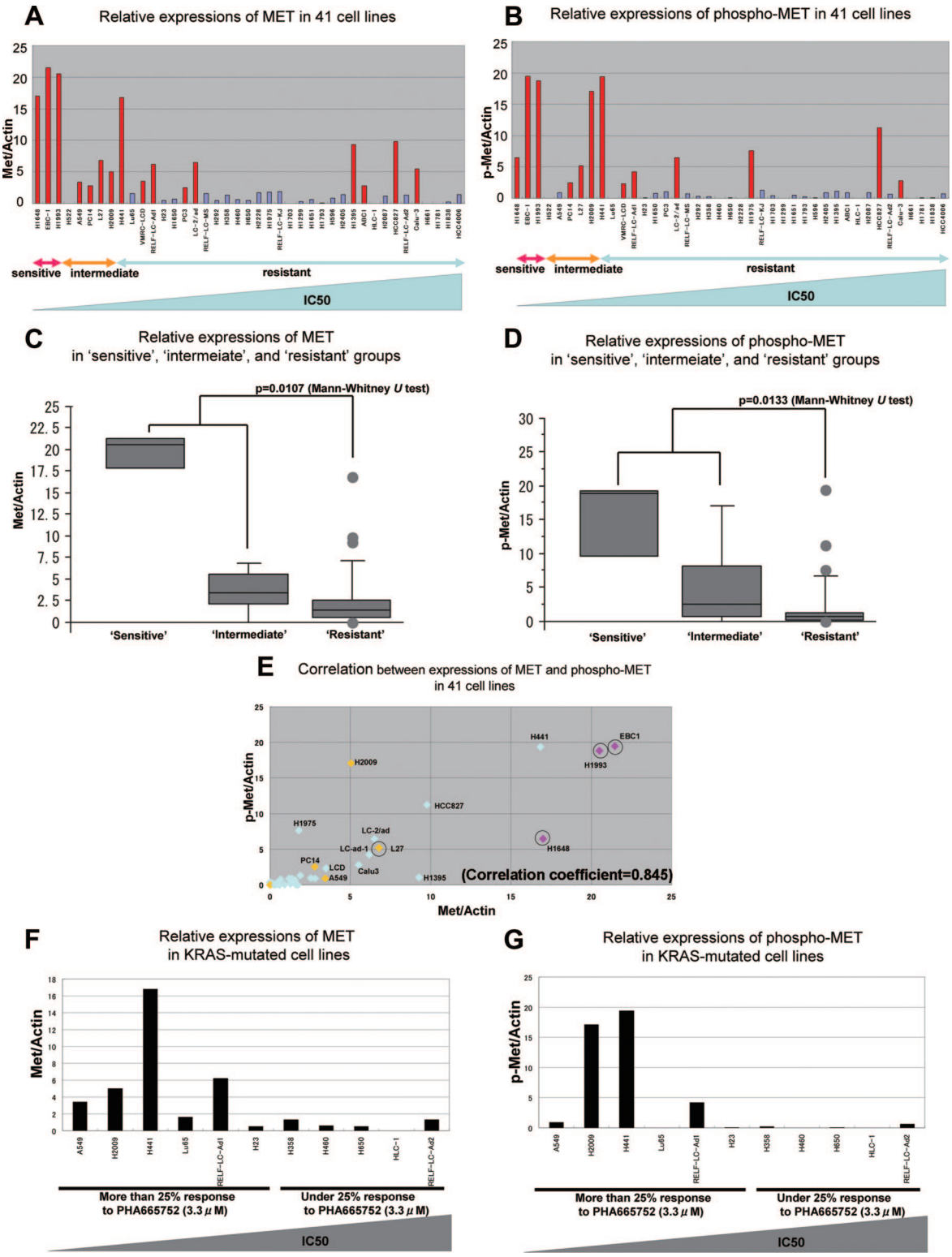

FIGURE 3. Expressions of MET and phospho-MET and their relationships with sensitivities to the MET inhibitor. The expressions of MET and phospho-MET were determined by Western blot analysis. The results were corrected for beta-actin and expressed in arbitrary units. *A*, Expression of MET in 41 cell lines. The protein expression of MET was consistent with the mRNA expression of MET determined by oligonucleotide array analysis (not shown). Cell lines were arranged in ascending order of their IC_{50} values. MET was strongly expressed in 16 cell lines (MET/beta-actin >2.0, red bars). *B*, Expression of phospho-MET in 41 cell lines. Phospho-MET was strongly expressed in 13 cell lines (p-MET/beta-actin >2.0, red bars). *C* and *D*, Box and

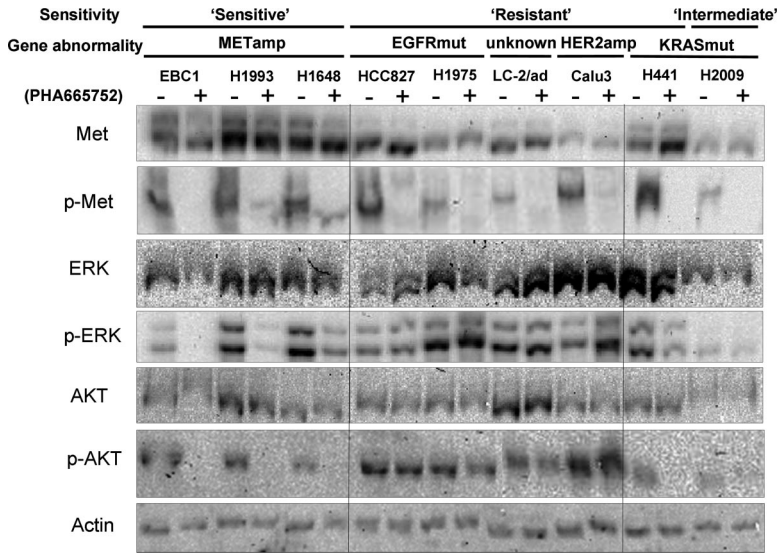

FIGURE 4. The effects of PHA665752 on the phosphorylation levels of MET, ERK, and AKT. Nine cell lines with high phospho-MET expression consisted of three sensitive cell lines (EBC1, H1993, and H1648), five resistant cell lines (HCC8227, H1975, LC-2/ad, Calu3, and H441), and one intermediate cell line (H2009). MET dephosphorylation was seen in all the cell lines tested. Dephosphorylation of ERK and AKT were seen in three of the three MET-amplified cell lines (EBC1, H1993, and H1648) and one of the two KRAS-mutant cell lines ("resistant" H441) but not in the four resistant cell lines (HCC8227, H1975, LC-2/ad, and Calu3).

in the sensitive and intermediate cell lines than in the resistant cell lines (MET: $p = 0.0107$, p-MET: $p = 0.0133$, Mann-Whitney U test) (Figures 3*C*, *D*).

Interestingly, the 13 cell lines in which high level of phospho-MET expression (p-MET/beta-actin >2.0) was found included not only 4 cell lines harboring MET amplifications but also 4 cell lines harboring KRAS mutation (H2009, H441, VMRC-LCD, and RELF-LC-Ad1), 3 cell lines harboring EGFR mutation (PC14, H1975, and HCC8227), and 1 cell line with HER2 amplification (Calu3). These results suggest that sensitivity to the MET inhibitor correlates with phospho-MET, whereas a high level of phospho-MET expression does not always predict good sensitivity to the MET inhibitor.

Modest Inhibitory Effect of PHA665752 in KRAS-Mutated Cell lines with High Levels of MET or Phospho-MET Expression

Close examination of the responses to PHA665752 at 3.3×10^{-6}M in Figure 1 revealed that the KRAS-mutated cell lines behaved somewhat differently from the EGFR-mutated and HER2-amplified cell lines. The EGFR-mutated and HER2-amplified cell lines with high level of phospho-MET (H1975, HCC8227, and Calu-3) showed only minimal responses (<25% inhibition) at 3.3×10^{-6} M PHA665752. In contrast, the KRAS-mutated cell lines with high levels of MET or phospho-MET (H2009, H441, and RELF-LC-Ad1) responded modestly at 3.3×10^{-6} M PHA665752 (Figures 3*F*, *G*). Meanwhile, the KRAS-mutated cell lines with low levels of MET or phospho-MET were wholly resistant to PHA665752 (Figures 3*F*, *G*). These results suggest that the MET inhibitor may have a modest inhibitory effect on KRAS-mutated lung cancer cell lines that strongly express MET or phospho-MET.

Effects of PHA665752 on MET-Dependent Signaling

ERK and AKT are important downstream effectors of EGFR and MET, and the effectiveness of gefitinib is correlated with the inhibition of phospho-ERK and phospho-AKT.[15] To elucidate the correlation between the effectiveness of PHA665752 and the inhibition of the phosphorylation of MET, ERK, and AKT, we tested the effect of PHA665752 treatment on ERK and AKT signaling in a subset of cell lines showing high phosphorylation of MET but variable sensitivities to the MET inhibitor PHA665752. This subset consisted of three sensitive cell lines (EBC1, H1993, and H1648), five resistant cell lines (HCC827, H1975, LC-2/ad, Calu3, and H441), and one intermediate cell line (H2009). The results are shown in Figure 4. PHA665752 (1×10^{-6} M) effectively suppressed the constitutive MET phosphorylation in all nine cell lines tested. Significantly, treatment with this concentration of PHA665752 also effectively abolished the baseline phosphorylation of downstream effectors of growth factor receptors such as ERK and AKT in all three sensitive cell lines with MET amplification (EBC1, H1993, and H1648). In contrast, PHA665752 had no effect on the baseline phosphorylation of ERK and AKT in four of the five resistant cell lines (HCC827, H1975, LC-2/ad, and Calu3); and somewhat paradoxically, it activated ERK in Calu3. This indicates that

FIGURE 3. (*Continued*) whisker plots of MET (*C*) and phospho-MET (*D*) expression levels in the sensitive, intermediate, and resistant groups. *E*, Correlation between MET and phospho-MET expression in 41 cell lines. The results are shown in a two-way scatter plot in which the *x* axis indicates the expression of MET corrected for beta-actin and the *y* axis indicates the expression of phospho-MET corrected for beta-actin. Purple, orange, and light blue rhombuses indicate "sensitive," "intermediate," and "resistant" cell lines, respectively. Cell lines harboring MET amplification are surrounded by black circles. *F* and *G*, Expressions of MET (*F*) and phospho-MET (*G*) in 11 KRAS-mutated cell lines. Cell lines were arranged in ascending order of their IC_{50} values.

ERK and AKT are activated through an alternative signaling pathway such as EGFR, HER2, or other growth driver in these resistant cell lines.

PHA665752 conferred a modest suppressive effect on ERK and AKT in the two KRAS-mutated cell lines H2009 and H441. This finding was consistent with the PHA665752 sensitivities observed in H441 and H2009 at 3.3×10^{-6} M in Figure 1.

Correlation Between MET and EMT Markers: E-Cadherin and Vimentin

EMT is associated with resistance to erlotinib.[16] It remains unclear, however, whether a similar association exists for MET inhibitor as well. To clarify this issue, we extracted gene expression data of E-cadherin and vimentin from the oligonucleotide array analysis data. With this data, we examined the correlation between expressions of MET and phospho-MET and expressions of E-cadherin and vimentin. E-cadherin gene expression showed modest positive correlations with phospho-MET expression (correlation coefficient = 0.482) and with MET expression (correlation coefficient = 0.361). Vimentin expression showed a modest negative correlation with MET expression (correlation coefficient = −0.365) and a weak negative correlation with phospho-MET expression (correlation coefficient = −0.266). These results suggest that the expression and activation of MET may be high in cell lines with epithelial phenotype but lower in cell lines with EMT.

Next, we examined the relationships among[1] the expressions of E-cadherin and vimentin;[2] the sensitivities to MET inhibitor; and[3] the genetic statuses of MET, EGFR,

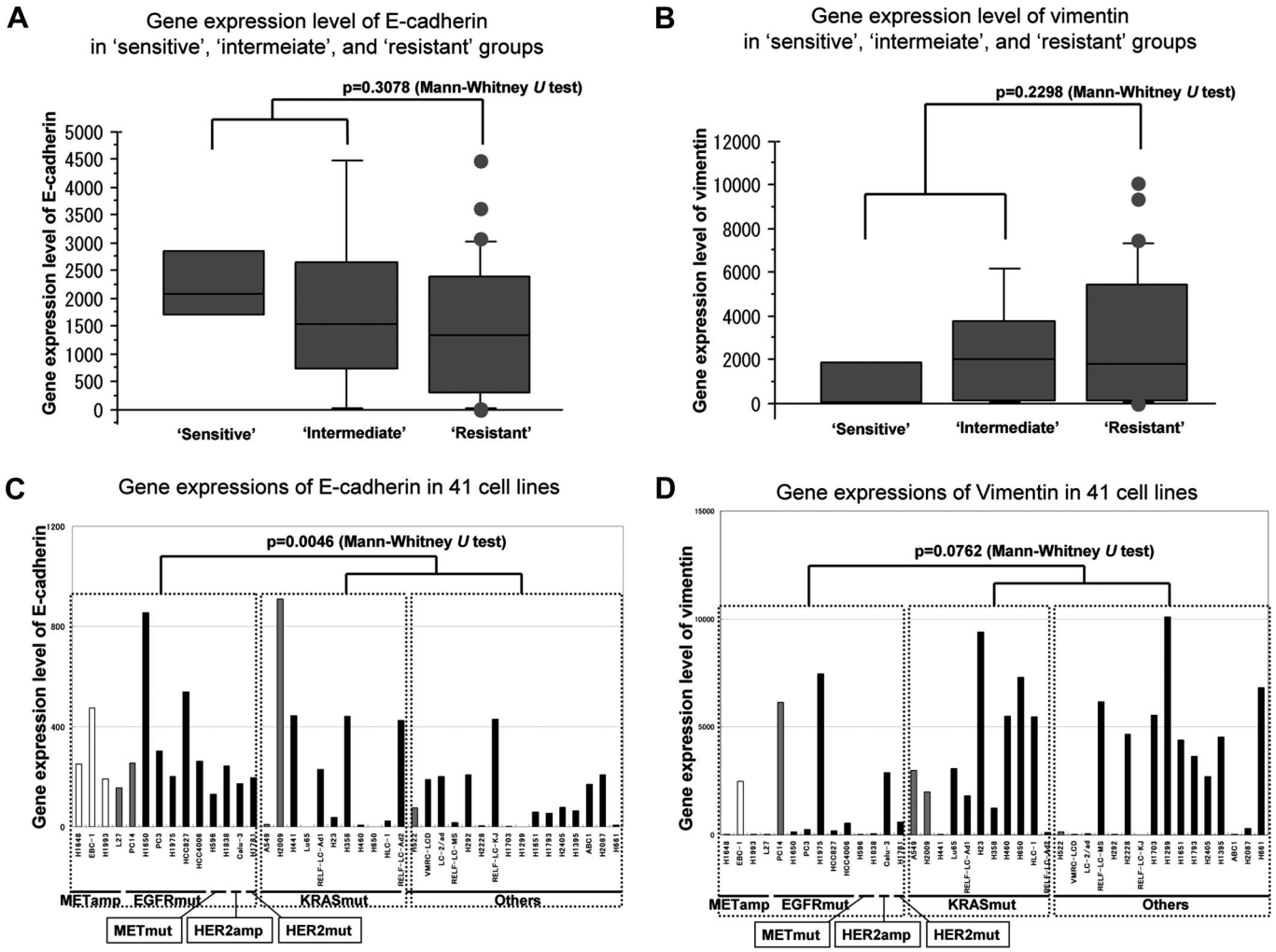

FIGURE 5. Expressions of E-cadherin and vimentin and their relationships with sensitivities to the MET inhibitor. *A* and *B*, Box and whisker plots showing the distribution of E-cadherin (*A*) and vimentin (*B*) gene expression levels in the sensitive, intermediate, and resistant groups. *C* and *D*, Expressions of E-cadherin (*C*) and vimentin (*D*), their relationships to the genetic statuses of MET, EGFR, HER2, and KRAS, and their sensitivities to the MET inhibitor. White bars, brown bars, and black bars indicate sensitive, intermediate, and resistant cell lines, respectively. Cell lines were arranged according to their genetic abnormalities as shown in the lower panel. METamp, MET amplification; EGFRmut, EGFR mutation; METmut, MET mutation; HER2amp, HER2 amplification; HER2mut, HER2 mutation; KRASmut, KRAS mutation.

HER2, and KRAS. The sensitivity to PHA665752 was not significantly correlated with the E-cadherin expression ($p = 0.3078$, Mann-Whitney U test) (Figure 5*A*) or vimentin expression ($p = 0.2298$, Mann-Whitney U test) (Figure 5*B*). This could be explained by significantly higher expression of E-cadherin in cell lines with genetic abnormalities of the ErbB family and MET than the cell lines without the genetic abnormalities of these kinases ($p = 0.0046$, Mann-Whitney U test) (Figure 5*C*). An inverse trend was observed for vimentin as well ($p = 0.0762$, Mann-Whitney U test) (Figure 5*D*).

DISCUSSION

In this study, we examined a panel of non-small cell lung cancer cell lines to identify the genetic statuses of MET, EGFR, HER2 and KRAS; the levels of MET expression and activation; and the expressions of EMT markers (E-cadherin and vimentin). Next, we used our results to determine whether these parameters were correlated with susceptibility to the MET inhibitor, PHA665752. We found that MET amplification was an excellent molecular marker for susceptibility to PHA665752 in lung cancer cell lines as previously reported.[8] However, our analyses also showed that MET amplification was not the sole determinant of sensitivity to PHA665752 or of high MET activation. There were several non-MET–amplified cell lines with intermediate sensitivities to PHA665752 and/or with high phospho-MET. EGFR mutation and MET amplification have both been reported to activate MET.[17] Our findings are consistent with this report and suggest that MET activation may depend not only on MET amplification or EGFR mutation but also on HER2 amplification or KRAS mutation in some cases.

It was interesting to note that the KRAS-mutated cell lines with high levels of MET or phospho-MET showed modest responses at 1.0 to 3.3×10^{-6} M PHA665752, even though both were deemed to be either intermediate or resistant. We also showed that the phosphorylation of ERK and AKT were inhibited by PHA665752 in KRAS-mutated cell lines with high phospho-MET. We presume that the maintenance of optimal cell growth and survival in these cell lines is dependent not only on the oncogenic mutant KRAS but also on MET activation. Small pharmacologic inhibitor is not currently available for the treatment of KRAS-mutated tumors. Our results support the view that a subset of KRAS-mutated tumors may be candidates for molecularly targeted therapy with MET inhibitors, as previously suggested from the study using mutant KRAS-induced mouse lung adenocarcinomas.[18]

Somatic exon 14 deletion of MET has been detected in resected non-small cell lung cancer.[19–21] This mutation seems to confer ligand-independent MET activation, and tumors harboring the mutation are potential candidates for anti-MET therapy.[21] In our data, two MET-mutated cell lines (H596 and H1838) exhibited low-level expressions of MET and phospho-MET and were resistant to PHA665752. Although relatively few MET-mutated cell lines were actually tested, our results clearly point to the need to further investigate the role of MET mutation, especially those resulting in exon 14 deletion, in lung cancer.

Our data also revealed a reverse correlation between MET expression and EMT. E-cadherin expression is reported to determine the in vitro sensitivity and predicted clinical activity of erlotinib in lung cancer patients.[16] In this study, however, we found that PHA665752 differs from erlotinib in that high expression of E-cadherin is not always correlated with the sensitivity to PHA665752.

In summary, we have shown that MET amplification is an excellent predictor of sensitivity to PHA665752 in lung carcinoma cell lines but not the sole predictor. Although less specific than MET amplification, high levels of MET and phospho-MET expression will also be useful as surrogate markers to select for sensitive tumors. High phospho-MET and the dependence of the AKT and ERK pathways on MET activation may predict sensitivity to PHA665752, especially in KRAS-mutated cell lines. Finally, our results will provide a rational basis for selecting lung cancer patients who may benefit from MET-targeted therapy.

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